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RESEARCH ARTICLE
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Toxicity Estimation of Graphene Nanoparticles in *Mus Musculus* Using Multiple Biomarkers

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Abstract
Graphene, a strong, super elastic, conductive, and crystalline carbon allotrope, is used in gene and drug delivery, osteon and dental implants, tissue grafting, biosensing, bioimaging, and photothermal therapies, etc. In the current study we evaluated the mutagenic potential of graphene by using Ames tests. Genetic damage and Hemolytic potential was also tested using comet assay and hemolysis assay. Male albino mice (n=66) were randomly selected, 5ml fresh blood from two of them were drawn for Hemolysis assay, and the remaining mice were divided into four groups; three treatment groups (dosages of 110mg/Kg, 220mg/kg, and 330mg/kg) and a control group. After treatment, blood was drawn for comet assay. Each group was again subdivided into three sub groups which were treated with L ascorbic acid (0.025 mg), retinol (0.03 mg) and no treatment for 15 days, and at 16th day same procedure was utilized for sample collection for comet assay. Data were recorded in CASP software and analyzed by using one-way ANOVA. The results of the Ames test indicate graphene is non-mutagenic, with a mutagenicity index less than 2 and 1.8 for TA98 and TA100 strains respectively. Dose dependent increase in hemolysis rate was also found in graphene because it can rupture the membrane of red blood cells (erythrocytes). Comet assay results showed Short-term oral exposure to graphene in male albino mice causes DNA damage, which can be repaired if exposure is ceased and some nutritional supplements if added.

Keywords: Gel electrophoresis, head DNA percentage, tail length, tail moment, hemolysis

INTRODUCTION

Graphite, a naturally occurring substance that has been used in human life for hundreds of years, is the source of graphene that is one atom thick, single layer of graphite that serves as the fundamental structural component of other allotropes include charcoal, fullerenes, and carbon nanotubes. Additionally, it is a crystalline allotrope of carbon that is robust, extremely elastic, conductive, and tightly bonded in a hexagon-shaped honeycomb lattice structure with sp2 hybridization. Two successive atoms in graphene are spaced around 1.42 Å apart (Park et al., 2020). Special chemical reactivity is present in graphene at the highest ratio of edgy carbons. Graphene has an ultimate tensile strength of 13x1010 Pascals. It is a material with unique physiochemical characteristics, including high surface area and exceptional electrical and thermal conductivities. It is very light at

97.3% transparent, stretchable up to 20% of its original length, and absorbs 2.3% of white light (Radadiya, 2015).

Its exciting applications in biomedicine and technology, including drug delivery, gene delivery, biosensing, bioimaging, disease diagnosis, bacterial inhibition, antiviral materials, cancer targeting, tissue engineering, electrical stimulation of cells, and photothermal therapy, have attracted a great deal of attention in recent years. Graphene's compatibility, selectivity, and solubility in a biological system are enhanced by structural variation (Wang et al., 2011).

Recently, COVID-19 has been combated with carbon-based nanomaterials (Serrano et al., 2021). Graphene is the most recent material that is helpful in photothermal treatment (PTT) (Dash et al., 2021). Using photothermal therapy, cancer cells are killed. Materials based on graphene are used in organ regeneration and bone healing. Graphene can be used to desalinate seawater because it permits water to evaporate without allowing other liquids or gasses to escape the flask (Dahanayaka et al., 2020).

Graphene's numerous applications have drawn the attention of scientists in recent times. Due to its hydrophobic surface, it becomes hazardous when it comes into contact with the lipids in cell membranes (Chen et al., 2012). It also activate macrophages upon inhalation and leads to pulmonary edema following intravenous administration in addition graphene and its compounds have been reported to cause significant pulmonary inflammation. Additionally, various studies have also documented the formation of granulomas and thrombus (Li et al., 2016).

Genetic toxicology tests have become an essential requirement for all new chemical substances in advanced countries throughout the world due to their inherited effects, and carcinogenicity which is regarded as a main public concern (Nersesyan et al., 2016).

L Ascorbic acid (Vitamin C) and Retinol (Vitamin A) are one of the most significant water-soluble and fat-soluble micronutrients, as well as antioxidants. Various in vitro studies have explained that vitamins A and C guard against oxidative DNA damage. A sufficient vitamin consumption reduces the amount of DNA adducts, strand breaks, and chromosome abnormalities. In study to estimates made in lymphocytes, a diet rich in food and vegetables reduces DNA damage in vivo (Karmieczak et al., 2020).

Battery of genotoxicity detection protocols and assays have been developed and are extensively being used in risk assessment and biomonitoring investigations including chromosomal aberration, micronucleus assay and single-cell gel electrophoresis (Comet assay). Comet test is one of the most reproducible, affordable, and simple among others. It is also used to detect the genotoxic potential of medications at an early stage of drug development and is capable of identifying a broad range of genotoxic endpoints. Moreover, state that it is highly sensitive and can identify even minute damage to DNA (Mortelmans and Zeiger, 2000).

One often used short-term bacterial assay for detecting mutagenic potential of chemicals is the *Salmonella typhimurium* reverse mutation assay, commonly called Ames Test. The Salmonella strain used in the assay is pre-mutated; it lacks the capacity to produce histidine, an amino acid that is essential for colonization growth. The capacity to synthesize this amino acid indicates that the test substance has corrected the mutation if it can grow and form colonies without the addition of histidine demonstrating the mutagenic nature of the material under test. This test is frequently referred to as a "reversion assay" for this reason (Mortelmans and Zeiger, 2000).

The Comet assay (single-cell gel electrophoresis) identifies DNA fragments at the individual cell level. It is a quantitative assay and there are different parameters including DNA in the comet Head, Comet tail length, Head DNApercentage, Tail DNApercentage, Tail Moment, and Olive tail moment. It is used to assess chemical agents that carry genotoxic potential (Purcarea et al., 2022; Maña et al., 2023).

The rupturing of red blood cells (erythrocytes) and releasing their content (Cytoplasm) into surrounding fluid (Blood plasma) before their normal life span of 120 days is called Hemolysis. During hemolysis, erythrophagocytes dispose of damaged red blood cells. This prevents the

extracellular release of hemoglobin, detoxifies heme, and recycles iron in a linked metabolic pathway. Hemolysis may occur in vivo and in vitro. Our body is constantly destroying old or damaged red blood cells and replacing them with new ones. Red blood cell destruction is a normal, healthy process. The accelerated rate of red blood cell destruction than normal is called hemolysis. Many causes of hemolysis include defective red blood cells, immune system-related conditions, infections, medications, pregnancy complications, medical devices, treatments, poison, and toxins. (Vallelian et al., 2022; Maña et al., 2023)

Hemolysis assay is cheap, accessible, and simple to perform. If the compound causes hemolysis, the hemoglobin (along with other cellular constituents) is released into the supernatant. Spectrophotometric measurement of free hemoglobin is also used in hospital laboratories for the evaluation of hemolysis in samples from patients. Hemolysis may occur either in vivo (i.e., within the body) as an indication of a variety of diseases, or in vitro (i.e., outside the body) because of improper blood collection or subsequent inadequate sample handling. The assessment of hemolysis is an important tool in many fields, including healthcare technology development, drug development, medical diagnostics, and life science research (Saebo et al., 2023).

As graphene usage has been increasing in human health services, there is need for a comprehensive functional toxicity, mutagenicity, and genotoxicity evaluation using multiple organisms particularly mammals must be carried out. Thus, the current work examines the hemolysis time, mutagenic, and genotoxic potential of graphene and assesses the effects of retinol and L Ascorbic acid on the organisms capacity for DNA repair in male albino mice. The Comet assay, Ames test, and Hemolysis Assay were performed to assess genotoxic potential, mutagenic potential and hemolysis time.

MATERIALS AND METHODS

To determine the Genotoxic impacts of graphene and protective effects of L Ascorbic acid (vitamin C) and Retinol (vitamin A) on the repair capability of DNA after ceasing the exposure, a trial was conducted on male albino mice at the animal house of the department of physiology, Government College University Faisalabad, Pakistan. This study was done after getting the permission from Departmental Bioethical Committee. The Ames test (Reverse mutation assay) was used to assess graphene's mutagenic potential. Hemolysis Assay and single-cell gel electrophoresis (Comet Assay) were used to assess genotoxicity.

Mutagenicity analysis of Graphene: Prior to conducting experiments, the strain's affectivity was assessed using 100µL of autoclaved distilled water as a negative control. For TA98 and TA100, 10µg/plate of potassium dichromate and 10µg/plate of sodium azide were utilized as positive controls. After creating the tubes with 2 ml of top agar, they were autoclaved. Melted top agar (45°C) was used for this purpose. After preparing a 0.5 mM histidine solution, filters were sterilized. This solution was divided into 200µL and added to the upper agar tubes. Subsequently, 100µL of a test strain *Salmonella Typhimurium* culture that had grown over night was added. The culture contained roughly 3x107 cells/ml (OD 0.7-0.8 at 590 nm), and the tube was vortexed slowly before being immediately put onto the minimum medium (V.B) plates. Following that, these tubes were filled with various graphene dilutions, including 1:20, 1:30, 1:40, and 1:80, and. Following a 48-hour incubation period at 37°C, revertant colonies were also observed on the plates (Ames et al., 1975). TA98 and TA100 were then used to examine every sample.

Mutagenicity Index (M.I) was calculated by the formula given below:

Mutagenicity Index (M.I) = No. of Revertant colonies in test plate/ No. of Revertant colonies in negative control plate.

The Test sample was considered mutagenic if the M.I. with TA98 was 2.00 and TA100 was 1.80.

***Animals*:** In the current study Swiss albino mice (*Mus musculus*, average weight; 27g±2.85, 4-6 weeks old) were used. For this trial, 66 male mice were chosen at random. 5 ml fresh blood was drawn from 2 mice in heparin tubes for hemolysis assay. The remaining 64 male mice, that were chosen at random, were housed in separate cages and split into four equal groups (one for control, and three for treatment). Normal laboratory conditions were adjusted for animals to provide them with proper care and lower the risk of any pain or stress. The temperature was maintained at 20-22 C, with a humidity level of 50-60 % and a light and dark period of 12/12h. Mice were provided free access to *ad laibita*. All animals were kept under critical observation during a whole trial period.

Graphene: Commercially available graphene nanopowder was used in this study that was purchased from the Richest group of Shanghai Ruizheng chemical technology company with an average size of 1nm.

Treatments: Three different doses of graphene were prepared based on the LD50 of graphene. The dosage consists of a low dose of 110 mg/kg, an intermediate effective dose of 220 mg/kg, and a maximum effective dose of 330 mg/kg. The dose was prepared by mixing the appropriate quantity in feed daily and administered orally for three consecutive days. The standard supplies of food and drink were given to the control group. After treatment, 2 mL of peripheral blood was drawn in sterilized EDTA tubes from 4 mice from each group and was immediately subjected to comet assay. Comet assay was performed according to (Hussain et al., 2022) with few modifications. The remaining 12 mice from each group were given a recovery period of 15 days during that period 4 mice from each group were treated with L ascorbic acid at the dose of 0.025mg/mice, 4 were given retinol at the dose of 0.03mcg/mice and remaining were not given any specific treatment. Normal water and food were supplied to all mice during the whole study period. On the 16th day, 2mL peripheral blood was drawn from each mouse by vein rupture via sterile disposable syringes in sterilized EDTA tubes for the comet assay.

comet assay:

The comet assay was carried out according to the protocol of Hussain et al., 2022 with slight modifications. Accordingly, one end frosted slides (pre-dipped in methanol) were burnt on blue flame for cleaning. 1ml of 1% normal melting point agarose was poured on the non-frosted portion of the slides and were allowed to dry overnight. For encapsulation, 100µl blood was mixed with 200µL of 0.5% Low Melting Point (LMP) agarose in an Eppendrof tube. Then this mixture was evenly distributed on a pre-coated slide with the help of a coverslip. Slides were placed on an ice bag for 5-10 min for polymerization. Then coverslips were removed gently. Slides were kept in freshly prepared Lysing Buffer for 1hr at 4°C in dark container. After that slides were placed in alkaline buffer (300 mM NaOH and 1mM EDTA) at pH 13.5 for 20 min at room temperature to allow DNA unwinding. Electrophoresis was conducted in the cold electrophoresis solution in horizontal electrophoresis tank in dark area for 20min at 25V and 300mA. Then slides were washed with neutralization buffer (pH 7.5) for five minutes, using fresh buffer every time step was performed thrice. Alcohol was poured on the sides for five minutes for fixation and allowed to dry. After neutralization and drying, slides were stained by using 75 µL of 5mg/mL of ethidium bromide and left overnight after covering with a cover slip. After staining, coverslips were removed from the slides, and observe slides under an Epifluorescent microscope at 40x magnification (Lobomad, Germany) with an excitation filter of 330-385 and an emission of 420nm by using comet software (CASP), 50 cells were counted on each slide(Hussain et al., 2021). CASP software was used to determine the various parameters, including Head length, Tail length, Comet length, Head DNA (%), Tail DNA (%), Tail moment (TM), and Olive Tail moment (OTM). The data was compiled using Excel. One-way ANOVA in SPSS software was used to statistically analyze the effects of

different treatments on seven parameters at ($P<0.05$). Tuckey's test was performed post hoc to compare the means within different treatment groups.

Hemolysis Assay:

For hemolysis 5ml of fresh blood was taken from 2 untreated healthy mice in the Heparin tube to avoid coagulation. The blood sample was centrifuged at 3000 rpm for 20 min, and the plasma was discarded according to safety rules. The remaining blood cells were washed three times with a double volume of phosphate buffer solution (PBS) to blood cells (pH 7.4). The stock solution of graphene was made 5mg/ml and serial dilution of test sample was as followed 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, and 1mg/ml respectively. For negative control, 1ml PBS was added to blood cells, and for positive control, 100ul 4%, triton X-100 was used. We had taken 400ul blood cells and 1ml PBS in each Eppendrof tube, and a 100ul graphene test sample was added to make the final volume. All samples were incubated at 37 °C for 1 hour and after that placed in the 60s on an ice bath. Then, again centrifuged at 3000rpm for 5 minutes. The supernatant was used to take absorbance (Abs) for the quantification of hemoglobin in samples at 540nm (Bhattacharya et al., 2014; Singh, et al 2020).

$$\text{Hemolysis percentage} = \frac{\text{Abs (Sample)-Abs (Negative control)}}{\text{Abs (Positive control) - Abs (Negative control)}} \times 100$$

RESULTS

The number of reverted colonies per plate for graphene in the TA98 bacterial strain are 968±122, 850±153.6, 620±106, and 611±8.49 at 1:20, 1:30 1:40, and 1:80 dilutions respectively. The number of reverted colonies per plate for graphene in TA100 bacterial strain is 931±118, 820±149, 760±128 and 710±7.07 at 1:20, 1:30 1:40, and 1:80 dilutions respectively (Table 1).

Table 1. No. of Reverted colonies per plate for TA98 and TA100 (strains of *Salmonella typhimurium*) incubated with different dilutions of graphene for 48 hrs.

Dilutions of graphene	1:20	1:30	1:40	1:80	Negative control	Positive Control
TA 98	968±122	850±153.6	620±106	611±8.49	608±12.02	1863±210
TA100	931±118	820±149	760±128	710±7.07	677±.53	2193±186.7

The mutagenicity index of graphene for TA98 at dilutions 1:20, 1:30 1:40, and 1:80 is 1.59, 1.39, 1.01, and 1.004 respectively. The mutagenicity index of graphene for TA100 at 1:20, 1:30 1:40, and 1:80 dilutions are 1.37, 1.21, 1.12, and 1.04. For negative control, its value is 1 at TA98 and TA100 bacterial strain, and for positive control, its value is 3.06 and 3.22 at TA98 and TA100 bacterial strain as shown in (Table 2). Graphene is non-mutagenic because the mutagenicity index of graphene is less than 2 for the TA 98 bacterial strain and less than 1.8 for the TA100 bacterial strain.

Table 2. Mutagenicity Index calculated for TA 98 and TA 100 strains of *Salmonella typhimurium* incubated with different dilutions of graphene for 48 hrs.

Dilutions	1:20	1:30	1:40	1:80	Negative control	Positive Control
TA 98	1.59	1.39	1.01	1.004	1	3.06
TA100	1.37	1.21	1.12	1.04	1	3.22

The genotoxicity and repair capacity of graphene in *Mus musculus* were assessed using comet assay. The comet assay results are displayed in Table 3. To evaluate the DNA damage caused by graphene, seven parameters were assessed: Head length (L Head), Tail length (L Tail), Comet length (L Comet), Head DNA percentage, Tail DNA percentage, Tail Movement (TM), and Olive Tail

Movement (OTM). The mean values of these parameters indicate that DNA damage was induced by graphene at all dose. The comparison of all the parameters across the various treatment groups is presented in Figure 1.

Different comet parameters in the blood of male albino mice that were exposed to different concentrations of graphene presented in Table 3 indicate that the L Head, L Tail, L Comet, Tail Movement (TM), and Olive Tail Movement (OTM) are non-significant and Head DNA percentage , Tail DNA percentage, are highly significant.

Different comet parameters among control and treated groups in male albino mice, which were exposed to different concentrations of graphene, are presented in Figure 1. It indicates an increase in L Tail, L Comet, Tail DNA percentage, TM, and OTM and a decrease in L Head and Head DNA percentage in the blood of male albino mice of graphene administration groups as compared to the control group.

Table 3. Comet assay parameters in the blood of male albino mice exposed to different concentrations of graphene

Parameters/ Factors	Control Group Mean ± S.D	Low Dose 110mg/kg Mean ± S.D	Medium Dose 220mg/kg Mean ± S.D	High Dose 330mg/kg Mean ± S.D	Significance level
LHead	45.64±1.47a	40.32±1.28a	34.42±1.42a	32.52±.22a	.099
LTail	12.36±2.03a	18.68±.19a	25.58±.50a	29.48±.02a	.254
LComet	58.40±.42a	59.00±1.66a	60.00±1.64a	62.00±.08a	.113
Head DNA percentage	96.40±.73a	93.15±.95bc	91.15±.68cd	88.85±.22d	.002
Tail DNA percentage	4.59±.18a	6.84±.25ab	9.165±.17b	10.64±0.21c	.003
TM	0.54±.05a	0.91±.08a	1.00±.01a	1.01±.06a	.361
OTM	0.98±.03a	1.10±.026a	1.24±.046a	1.25±.069a	.927

p>0.05= Non-significant, p<0.05= significant, p<0.01 = Highly Significant. Mean Sharing the same letters in a row is statistically non-significant.

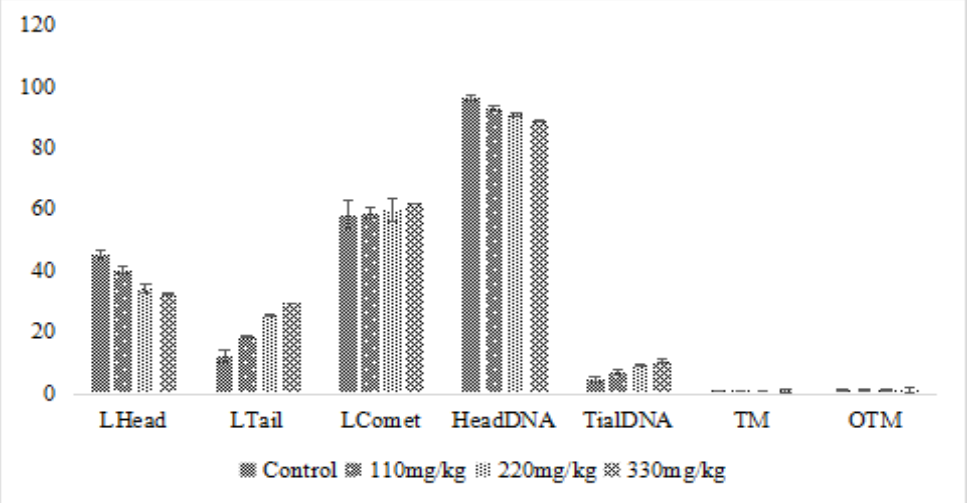

Figure 1. Comet assay parameters in the blood of male mice exposed to different concentrations of graphene.

Different comet parameters in the blood of male albino mice, which were exposed to L-ascorbic acid (Vitamin C) in (Table 4) indicate that the L Head, L Tail, L Comet, and Tail Moment, are highly significant and the Head DNA percentage, Tail DNA percentage, OTM, are non-significant whereas results of mice exposed to Retinol (Vitamin A) indicated that L Head, Head DNA percentage, Tail DNA percentage are highly significant, L Tail, L Comet, OTM are non-significant, and Tail Moment is significant. Different comet parameters in the group which were not exposed to any special dose, indicate that Head DNA percentage and Tail DNA percentage are significant. L Head, L Tail, L Comet, Tail movement (TM), and Olive tail movements (OTM) are non-significant.

Table 4. Comparison of comet assay parameters in blood of male albino mice treated with L ascorbic acid, Retinol, and without any special treatment after recovery period, previously exposed to different doses of Graphene.

	Parameters/Factors	**Control Group** Mean ± S.D	**Low Dose** Mean ± S.D	**Medium Dose** Mean ± S.D	**High Dose** Mean ± S.D	**Significance level**
L Ascorbic Acid	**LHead**	47.45±.31a	46.11±.31b	44.34±.16c	40.13±.18d	.000
	LTail	5.1±.65a	9.64±0.02ab	14.38±.12bd	21.62±0.28d	.009
	LComet	52.55±.33a	55.76±0.13a	59.22±.35b	61.75±.47c	.000
	Head DNA percentage	95.4±.68a	93.95±1.02a	93.69±.011a	93.09±.18a	.077
	Tail DNA percentage	4.42±.27a	5.10±.19a	5.61±.24a	6.82±0.14a	.081
	TM	0.48±0.0a	0.51±.00ab	0.63±.01b	0.84±.01c	.001
	OTM	0.76±.007a	0.83±0.02a	0.88±.07ab	0.89±.01b	.058
Retinol	**LHead**	46.60±.56ab	45.50±.70bc	43.50±.71c	40.50±.70d	.003
	L Tail	6.23±.000a	8.85±.08a	16.13±.16a	20.25±1.43a	.142
	LComet	52.83±.56a	54.35±.62a	59.63±.87a	60.75±2.14a	.102
	Head DNA percentage	95.84±.11a	94.31±.44bc	93.45±.31cd	92.61±.54d	.005
	Tail DNA percentage	4.06±.04ab	5.01±.02b	6.96±.00c	7.33±0.21d	.005
	TM	.45±.04a	.64±.07b	.64±.025cd	.71±.02d	.014
	OTM	0.693±.09a	.70±.044a	.77±.077a	.828±.0057a	.274
Without any special dose	**LHead**	46.07±1.19a	44.11±.162a	41.71±.042a	39.84±.55a	.121
	L Tail	7.41±.67a	11.28±1.3a	15.63±.57a	21.92±.67a	.439
	LComet	53.48±.51a	55.39±1.46a	57.34±.61a	61.76±1.2b	.231
	Head DNA percentage	95.94±.026a	94.61±.009b	92.62±.86bc	91.92±.98c	.016
	Tail DNA percentage	4.62±.009a	5.06±.081b	7.32±0.13c	7.99±.22c	.025
	TM	0.527±.036a	0.72±.007a	0.89±0.0a	1.00±0.0a	.116
	OTM	.867±.063a	0.9±.045a	0.914±.011a	0.951±.07a	.444

$p>0.05$= Non-significant, $p<0.05$= significant, $p<0.01$ = Highly Significant. Mean Sharing the same letters in a row is statistically non-significant.

Comparison of Head length in blood of male albino mice among three treatment groups L ascorbic acid group, Retinol group, and without any special treatment group which were previously exposed

to graphene is presented in Figure 2, showed Head length is increased in L ascorbic acid as compared to retinol group and without any special treatment group at control, low and medium dose but at high dose the effect of L ascorbic acid and retinol is nearly equal. It shows that the Head length is repaired by using the L ascorbic acid.

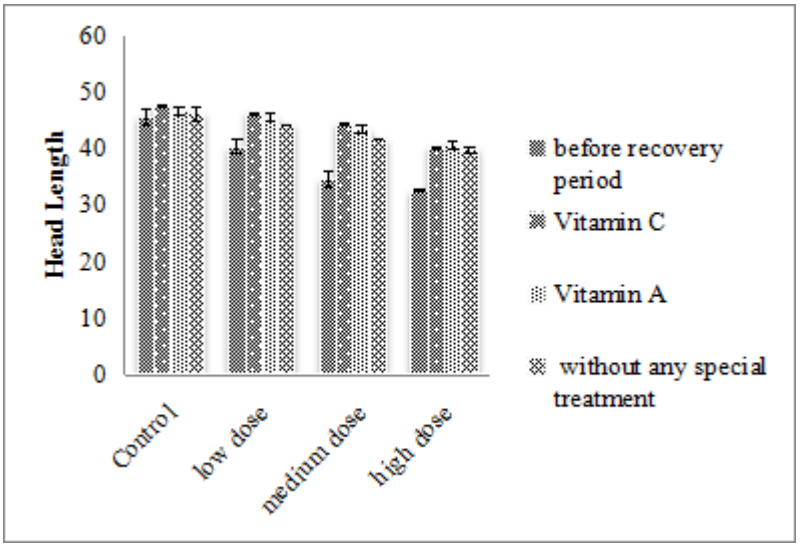

Figure 2. Comparison of Vitamin C, Vitamin A, and without any special treatment in Head length of male albino mice previously exposed to different doses of graphene.

Comparison of Tail length in blood of male albino mice among three treatment groups i.e. L ascorbic acid group, Retinol group, and without any special treatment group which were previously exposed to graphene is illustrated in Figure 3, showed that the value of Tail length is decreased by using the L ascorbic acid and retinol. Retinol is more effective at low and high doses than L ascorbic acid and L ascorbic acid is more effective than retinol in control and low-dose groups.

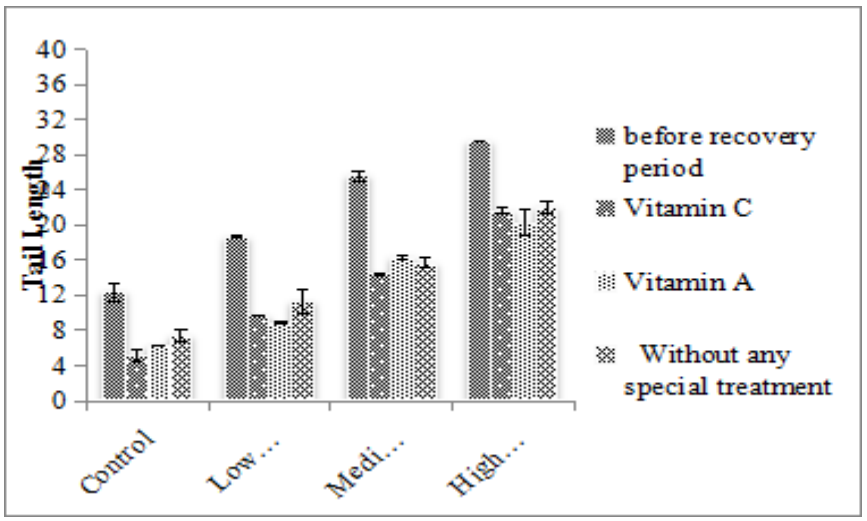

Figure 3. Comparison of Vitamin C, Vitamin A, and without any special treatment groups in Tail length of male albino mice previously exposed to different doses of graphene.

Comparison of comet length in the blood of male albino mice among three treatment groups L ascorbic acid group, Retinol group, and without any special treatment group which were previously exposed to graphene is obvious Figure 4, showed the effects of L ascorbic acid on L comet is more effective than retinol and to group without any special treatment at control group. The recovery

group without any treatment is more effective than other groups at medium doses. The effects of retinol on Comet length are more effective than other groups at low doses and high doses.

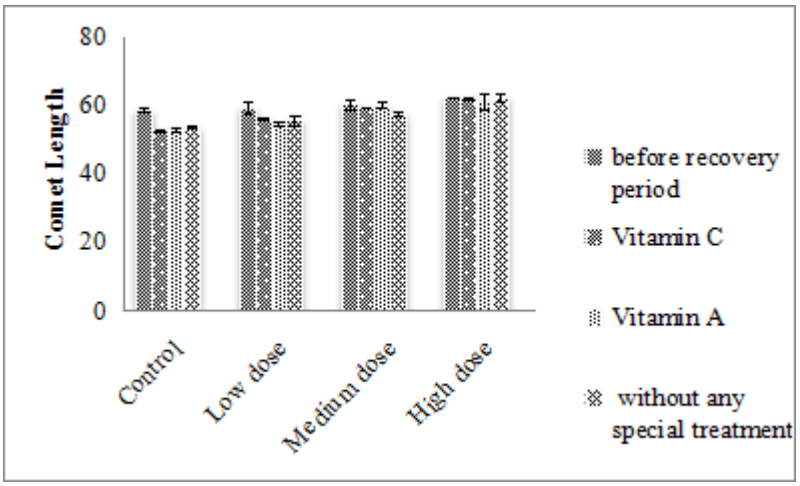

Figure 4. Comparison of Vitamin C, Vitamin A, and no specific treatment in Comet Length of male albino mice previously exposed to different doses of graphene.

Comparison of Head DNA percentage in the blood of male albino mice among three treatment groups L ascorbic acid group, Retinol group, and without any special treatment group which was previously exposed to graphene in (Figure 5), which showed that the Head DNA percentage is highly repaired by using L ascorbic acid than retinol and without any special treatment group at medium dose and high dose. The recovery period is more effective in control and low-dose groups.

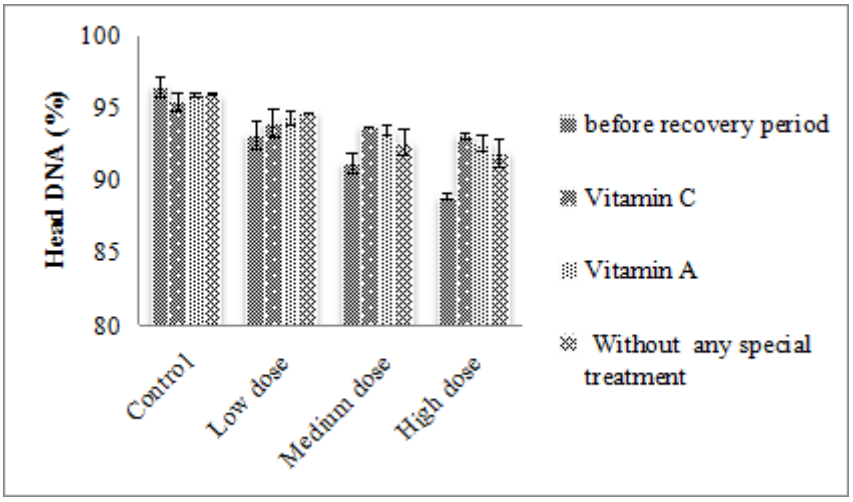

Figure 5. Comparison of Vitamin C, Vitamin A, and without any special treatment in Head DNA percentage of male albino mice previously exposed to different doses of graphene.

Comparison of Tail DNA percentage in the blood of male albino mice among three treatment groups L ascorbic acid group, Retinol group, and without any special treatment group which was previously exposed to graphene (Figure 6**)**, showed that L ascorbic acid treatment group gave a high rate of repair at medium and high dose groups than retinol and without treatment.

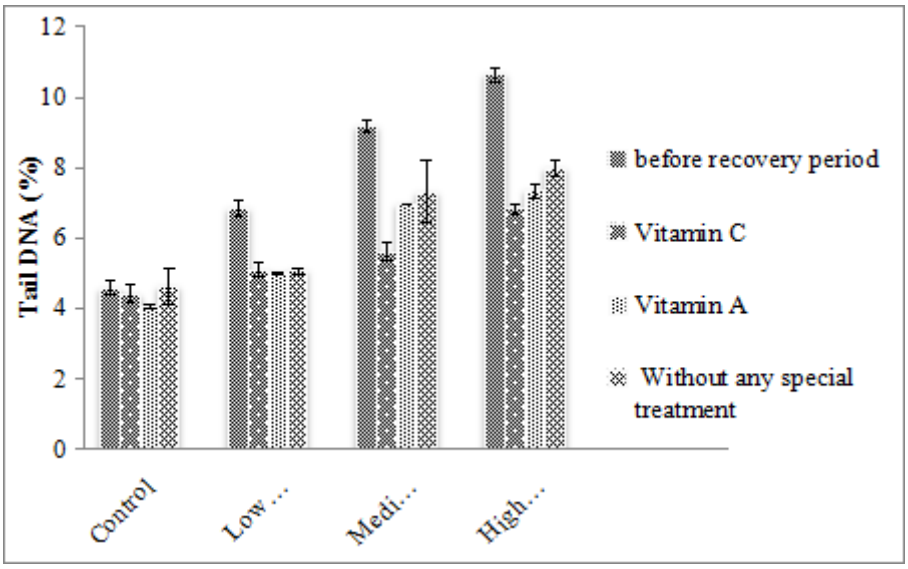

Figure 6. Comparison of Vitamin C, Vitamin A, and no specific treatment in Tail DNA percentage of male albino mice previously exposed to different doses of graphene.

Comparison of Tail Movement (TM) in the blood of male albino mice among three treatment groups L ascorbic acid group, Retinol group, and without any special treatment group that were previously exposed to graphene (Figure 7), showed that retinol is more effective than L ascorbic acid and no special treatment group at control group and high dose. At low and medium dosages, L ascorbic acid impose better repair than retinol and without special treatment.

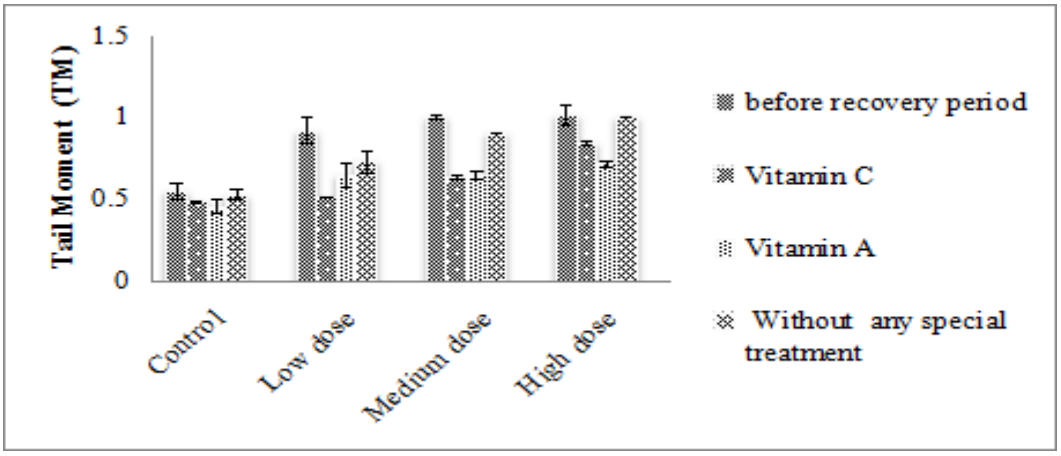

Figure 7. Comparison of Vitamin C, Vitamin A and without any special treatment in tail moment (TM) of male albino mice previously exposed to different doses of graphene.

Comparison of Olive Tail Movement (OTM) in the blood of male albino mice among three-treatment groups L ascorbic acid group, Retinol group, and without any special treatment group was previously exposed to graphene in Figure 8, showed that retinol is more effective than L ascorbic acid and without any special treatment group.

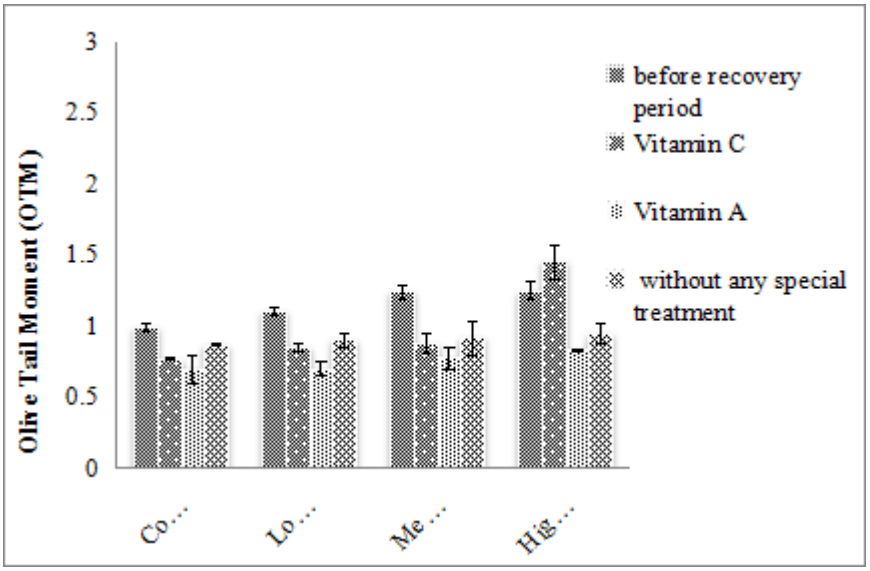

Figure 8. Comparison of Vitamin C, Vitamin A and without any special treatment in Olive tail moment (OTM) of male mice previously exposed to different doses of graphene.

To assess the hemolytic potential of nanomaterials, hemolysis assay was used. In all the graphene concentrations tested, the red blood cell lysis rate was greater than 5% (>0.05). The absorbance values of graphene were 0.354, 0.416, 0.454, 0.488 and 0.863, at 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml and 1.25mg/ml concentrations respectively. Dose dependent increase in hemolysis percentage was observed with maximum hemolysis (26.7%) at the concentration of 1.25mg/ml (Figure 9). The lack of hemolysis in the negative control group and almost 100% hemolysis rate in the positive control group confirmed the accuracy of the assay.

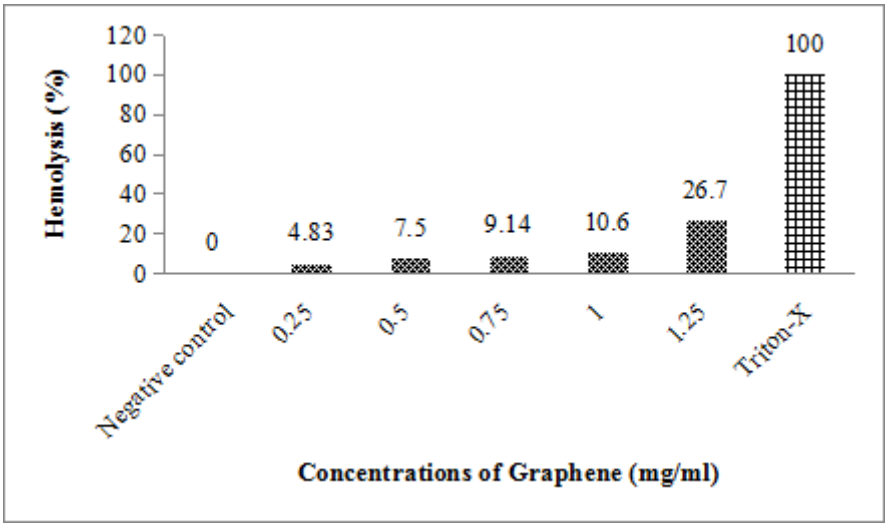

Figure 10. Comparison of hemolysis activity of PBS, Triton-X and Graphene at different concentrations (mg/ml) after 1 hour of incubation.

DISCUSSION

Different in vivo and in vitro studies are available in the literature where the toxicity of various environmental chemicals against multiple biological systems have been evaluated. In recent years the usage of graphene in biomedicine become high and taken the interest of different groups working on the toxicity of various environmental chemicals and they have found it phytotoxic (Begum et al., 2011), neurotoxic (Soares et al., 2017) cytotoxic (Nasirzadeh et al., 2019) and nephrotoxic (Foroutan et al., 2020). However comprehensive information about the mutagenic and

genotoxic effects of graphene in different mammalian systems is lacking. Genetic material has the capability of self-repair when exposure to a toxic substance is stopped, depending upon the nature and intensity of the damage. Sometimes the addition of certain micronutrients in the diet further enhances the repair process.

In present study, *Salmonella typhimurium* TA98 and TA100 bacterial tester strains were used to perform the Ames test (Bacterial Reverse Mutation test). Different dilutions of graphene were made. Positive and negative controls were also included in this test. Graphene is non-mutagenic because the mutagenicity index of graphene is less than 2 for the TA 98 bacterial strain and less than 1.8 for TA100 bacterial strain.

Lamponi, 2022 assessed the mutagenicity of graphene flake (GF) and aqueous graphene paste (AGP) at six different concentrations using the Salmonella mutagenicity assay. The S9 metabolic activator was tested in the presence and absence of the TA98 and TA100 strains. The samples do not show dose-response mutagenicity, as evidenced by the absence of rise in revertants per plate with increasing concentrations of GF and AGP for both TA98 and TA100 with and without S9 fraction. There were fewer revertants and a significant difference in number from the positive control at the highest dose of graphene examined (10000 μg/mL) ($p < 0.05$).

Zhang et al., 2022 reported that silver nanoparticles (AgNPs) at 100 to 500 μg/plate using four *S. typhimurium* strains, (TA98, TA100, TA1535, and TA1537) using Ames tests with or without S9 for 40−59 nm did not display a mutagenic effect.

Comet assay is widely used for monitoring genetic damage and repair. This study was used to monitor the genetic damage of graphene after oral exposure to albino mice. Albino mice are model organisms, widely used in physiological, pharmacological, and biomedical research due to their close resemblance with humans. DNA repair capability was also assessed and the protective effects of Vitamin C and Vitamin A on the repair capability of DNA were monitored too. In this study, we observed a significant difference in all parameters including Head length (L Head), Tail length (L Tail), comet length (L comet), Head DNA percentage, Tail DNA percentage, Tail movement (TM), and Olive Tail movement (OTM) of comet assay among different doses when compared with a control group. Maximum harm was noted at a high graphene dosage. It indicates DNA damage was increased by increasing the dose of graphene.

In our study, we found a significant difference in the DNA repair process between the groups who received L ascorbic acid (Vitamin C), retinol (Vitamin A), and without special treatment. The maximum repair was observed in L ascorbic acid-treated groups. Both vitamins (C&A) have protective effects and elevate the repair process. It indicates that vitamin C and vitamin A repair the graphene-induced DNA damage. It can be stated that graphene produces reactive oxygen species (ROS) that may be absorbed by vitamin C and vitamin A. Some studies have shown conflict with vitamin A (Murata and Kawanishi, 2000) that's why the effect of vitamin A was less than vitamin C.

The teratogenic impacts of GO nanosheets in the hemocyte cells of the flies (*Drosophila melanogaster)* were evaluated by using the comet assay. It revealed that exposure to GO nanosheets enhanced the amount of DNA damage in cells (Priyadarsini et al., 2019).

The relationship between the size of the graphene quantum dots and the DNA damaging mechanisms was investigated. The small GQDs penetrate the molecule of DNA and caused DNA base mismatch, although the larger GQDs joined to the two ends of the molecule of DNA and caused DNA unwinding (Kong et al., 2020).

The toxicity caused in bacteria due to graphene oxide nanosheets were evaluated after growing the bacteria in different concentration of GO solutions (0.004–1μg/μL). Graphene oxide nanosheets enter into the bacteria through puncture the cell walls and causing DNA damage. The toxicity of GO and rGO were compared by using comet assay in human retinal pigment epithelium ARPE-19 cells after 24h exposure to GO and rGO. Graphene oxide caused a lower level of DNA damage than reduced graphene oxide at high doses. It has been proposed that functional groups containing

oxygen are crucial in reducing genotoxicity. A comet assay that has been enzyme-modified is appropriate for estimating oxidative DNA damage (Ou et al., 2021).
Abdul Ghaffar et al., 2021 reported that there was a significant ($p < 0.05$) increase in the whole blood DNA content and the frequency formation of micronuclei in erythrocytes of fish that were exposed by glyphosate at higher concentrations.
In another study the genotoxicity and pathologic potentials of fibronil insecticide on *Labeo rohita* evaluated by (Abdul Ghaffar et al., 2021). He concluded that there is a significantly increased DNA contents in the blood of fish exposed to higher concentrations of fipronil may be due to the induction of oxidative stress and production of free radicals.
In Similar to (Hussain et al., 2021) who examined trichlorfon increases the frequency of micronuclei and erythrocytes with condensed nuclei development in exposed cocks. Moreover, in isolated blood cells, trichlorfon enhances the comet tail length and percentage of DNA damage While (Wang et al., 2022) evaluated that the frequency of DNA damage in lymphocytes and hepatocytes that treated with different concentration of pendimethalin was significantly ($P \leq 0{:}05$) higher as compared to control group. Higher frequency of morphological changes, nuclear alterations in red blood cells, and an increased risk of bisphenol A-induced DNA damage in the tissues of the brain, liver, kidneys, and gills were evaluated by (Akram et al., 2021).

Hussain et al., 2024 reported that there was a significantly increased the percentile rate of DNA damage in isolated cells of multiple tissues (gills, kidneys, liver, and brain) of grass carp as well as the frequency of formation of micronucleus in red blood cells in a time- and dose-dependent manner as compared to control group exposed by MgO NPs.
Demir and Marcos (2018) found contrasting results as compared to the present study. He assessed the cytotoxicity and mutagenicity of graphene and multiwalled carbon nanotubes (MWCNT) in the L5178Y/ Tk+/–3.7.2C mouse lymphoma cell line after exposures varying from 0.01 to 250 μg/mL. At doses of less than 250μg/mL, neither graphene nor MWCNT exhibited significant cytotoxic or mutagenic effects. Gene expressions, intracellular ROS, necrosis, apoptosis, and mutation frequency at the TK locus did not exhibit any discernible changes.
To assess the hemolytic potential of nanomaterials, hemolysis assay were used. The absorbance values of graphene are 0.354, 0.416, 0.454, 0.488, and 0.863 at 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml and 1.25mg/ml concentrations respectively. There were significant differences ($p>0.05$) between different concentrations. Hemolysis % was the highest for graphene is 26.7% at the concentration of 1.25mg/ml. The red blood cell lysis rate is greater than 5% (>0.05) which means graphene causes hemolysis are not suitable for human health services. Increased concentration of graphene resulted in increased hemolysis. The hemolytic activity of graphene oxide on red blood cells may be caused by the rupture of the cell membrane, the concentration of red blood cells, how long they have been stored, the health of the animal, the centrifugal speed, the exposure period, and the temperature during incubation. In similar to Dasmahapatra et al., 2019 graphene based materials have the ability to enter cells, penetrate through cell barriers, and interact with nearly every place within a cell, including the nucleus, cytoplasmic organelles, and plasma membrane. The genome and epigenome may be harmed by interactions with DNA.

Using in vitro hemolysis and WST-8 viability assays, (Liao et al., 2011) examined the hemocompatibility and cytotoxicity of graphene-based materials (graphene oxide (GO) and graphene sheets (GS)) of different sizes and oxygen content in suspended human erythrocytes and adherent dermal fibroblasts. On RBCs, all of the GO and GS show dose-dependent hemolytic activity. The hematological and histological changes in major carp (Catla catla) exposed to different concentrations of copper (Cu) and cadmium (Cd) were assessed by (Naz et al., 2021).Total number of white blood cells and neutrophils increased drastically, the number of red blood cells, hemoglobin (Hb), hematocrit (Hct), lymphocytes, and monocytes significantly reduced.

The protective effects of vitamin C on gastric epithelial cells SGC-7901 was observed that were infected by *Helicobacter pylori* and found that vitamin C diminishes ROS and can repair damaged DNA *H.pylori* infected SGC-7901 cells (Shi and Zhang, 2006).The role of vitamin C on reactive oxygen species (ROS) and DNA damage in lead-induced hypertension in male Wister rats was evaluated. ROS caused oxidative stress and DNA damage that is prevented by using Vitamin C (Attri et al., 2003). In similar to Parveen et al. (2014) showed that vitamin C (VC), at a dose of 500 mg/kg, may have had a protective effect against DNA damage and chromosomal aberrations caused by acute iron sulfate (FeSO4) injection in Wistar rats. The possible mechanisms of VC can be linked to either its indirect reduction of reactive oxygen species (ROS) or its ability to scavenge free radicals.

Contrasting results were found as compared to the present study. Vitamin A (Retinol) and its derivatives caused cellular DNA damage through superoxide generation and were detected by `pulsed-field gel electrophoresis (Murata and Kawanishi 2000).

Conclusion: This study concludes that graphene is non-mutagenic. It can damage the cell membrane of RBCs (erythrocytes) and DNA in the blood cells of mice at very low level denoting the genotoxicity of graphene that was dose and time-dependent is slightly. Graphene particles pass through the cell membrane and produce reactive oxygen species (ROS). ROS is proposed as the mechanism of the genotoxicity of graphene. L ascorbic acid and retinol have protective effects against DNA damage of graphene. Furthermore, in the future graphene must be taken into consideration and should be optimized and controlled by using L ascorbic acid and retinol in biomedical applications.

Conflict of Interest*:* This is original work and the authors have no conflicts with any research group or Funding agency. Moreover, this work is not submitted to any other journal for Publication.

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